

Fate of Model Xenobiotics in Calcareous Marine Algae¹

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ABSTRACT: Uptake, depuration, and metabolism of p-nitroanisole (PNA) and p-nitrophenol (PNP) were investigated in *Halimeda*, *Padina*, and *Porolithon* species, all of which are calcareous marine algae found in tropical waters. The algae were exposed to filtered seawater solutions of either PNA or PNP in a static system for 24 hr (uptake period), then placed in clean water and allowed to release absorbed chemical and possible metabolites for 24 hr (depuration period). Concentrations of the chemicals were monitored spectrophotometrically, and the water at the end of uptake and depuration was extracted onto a column of Amberlite XAD-4 resin, eluted sequentially with methylene chloride and methanol, and analyzed for metabolites by high-pressure liquid chromatography (HPLC). Results showed that the algae absorb PNA but not PNP. There was no indication that they were capable of metabolizing PNA, except inconsistently, to PNP. However, half of the absorbed PNA remained unaccounted for, and may either have been metabolized to undetected metabolites or bound to tissue macromolecules.

CALCAREOUS MARINE ALGAE are important components of reef structures. *Halimeda*, a member of the Chlorophyta, is a major sand producer in many areas (Goreau and Goreau 1973). *Porolithon*, a rhodophyte, helps maintain reef structure and integrity by forming algal ridges at the reef front, as well as by cementing together the calcified fragments that compose the reef itself (Goreau 1963, Goreau and Goreau 1973). Although members of the Phaeophyta are not known to contribute significantly to the building or maintenance of the reef structure, they are prominent members of the reef community. In our work, this third major division of calcareous macroalgae was represented by *Padina*, which was common at the study site, Hawaii Institute of Marine Biology on Coconut Island, Oahu, Hawaii.

Because many of these algae live on the shallow reef flats, they are likely to be exposed to xenobiotics introduced either indirectly in shoreline runoff or directly as chemical spills (pesticides, oil, etc.) on the reef. Although there have been studies on the ecology, growth, and intermediary metabolism of these algae (Hillis-Colinvaux 1980, Johansen 1981), there seem to be no reports on their ability to metabolize xenobiotic compounds. Xenobiotic metabolism consists of primary reactions (oxidation, reduction, and hydrolysis), and secondary reactions (conjugation to polar groups), which render the chemical more water-soluble (polar) and thus easier to excrete. Because it is moderately fat-soluble and it can be modified by many metabolic reactions, p-nitroanisole (PNA) is a good model pollutant; it can be demethylated via oxidation to form p-nitrophenol (PNP), ring oxidized at the 2-position to the nitrocatechol, or reduced to form an amine (Figure 1). In addition, the analytical techniques to detect and measure PNA and many possible metabolites have already been developed (Foster and Crosby 1986).

The purpose of this research was to investigate the uptake, depuration, and biodegradation of the model compounds in three types of

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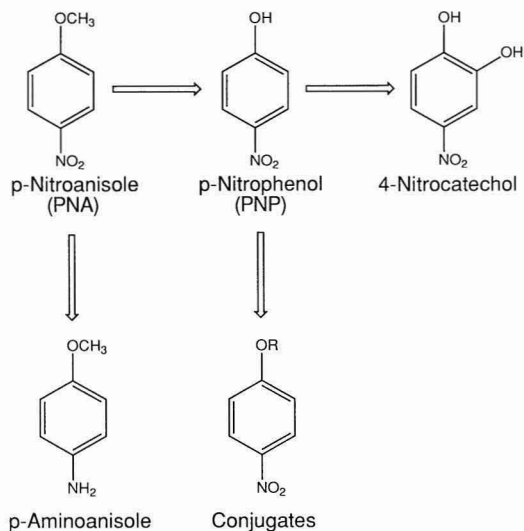


FIGURE 1. Possible metabolism routes of PNA and PNP (Foster and Crosby 1986). Conjugates may be sulfate ($R = \text{OSO}_3\text{H}$), glucoside ($R = \beta\text{-D-glucose}$), and galactoside ($R = \beta\text{-D-galactose}$).

algae. In case they were unable to demethylate the PNA into PNP, the ability to conjugate at the hydroxyl group could still be investigated by using PNP itself.

MATERIALS AND METHODS

Chemicals and Reagents

PNA and PNP, purchased from Aldrich Chemical Co., were recrystallized and determined to be pure by high-performance liquid chromatography (HPLC). Reference standards, including 4-nitrocatechol, PNP sulfate, PNP $\beta\text{-D-glucoside}$, and PNP $\beta\text{-D-galactoside}$, were purchased from Sigma Chemical Co. and used without further purification.

Algae

Algae were collected from Kaneohe Bay, Oahu: *Halimeda discoidea* was collected on Checker Reef, *Porolithon* sp. from Reef 43, and *Padina japonica* from the lagoon at the Hawaii Institute of Marine Biology on Coconut Island. The algae were kept in a flowing

seawater system in partial sunlight and were carefully cleaned of epiphytes and commensal animals just before each experiment. *Porolithon* algal heads were broken into small pieces, scrubbed with a brush, then placed in seawater, and purged with nitrogen gas for 15 min to remove any animals remaining within the calcareous matrix.

Metabolism Chamber

A 400-ml, uncovered widemouth jar was used as a metabolism chamber. For *Halimeda* and *Padina*, ca. 10–15 g wet weight were used for each experiment; for *Porolithon*, ca. 50 g wet weight was used. Algae were exposed to either PNA or PNP (2 mg/l, 350 ml, in $0.2\text{-}\mu\text{m}$ -filtered seawater) at room temperature ($26 \pm 2^\circ\text{C}$) and natural indoor lighting for a 24-hr uptake period. The algae then were rinsed and transferred to 350 ml of clean $0.2\text{-}\mu\text{m}$ -filtered seawater and allowed to release absorbed chemical and possible metabolites for 24 hr (depuration period). Water samples (5 ml) were taken for analysis throughout both uptake and depuration periods at ca. 1, 2, 4, 8, 12, and 24 hr. An identical jar, but containing no algae, was maintained simultaneously to control for the physical loss or abiotic degradation of either PNA or PNP.

Sample Analysis

All water samples were filtered before analysis to reduce analytical interference due to particulates, and the filters ($0.45\text{-}\mu\text{m}$ Nucleopore membrane filter) were tested to ensure that neither PNA or PNP were adsorbed (recoveries for both compounds were 100%). Water samples were analyzed using a Beckman Model DB-6 spectrophotometer; PNA was monitored by absorbance at 318 nm, and PNP at 401.5 nm. These values were corrected for instrument drift by subtracting a background value taken where neither compound absorbed (500 nm).

At the end of both uptake and depuration periods, the remaining water was extracted by passing through an Amberlite XAD-4 resin column ($2 \times 10\text{ cm}$). The column was rinsed with 100 ml of distilled water, then sequenti-

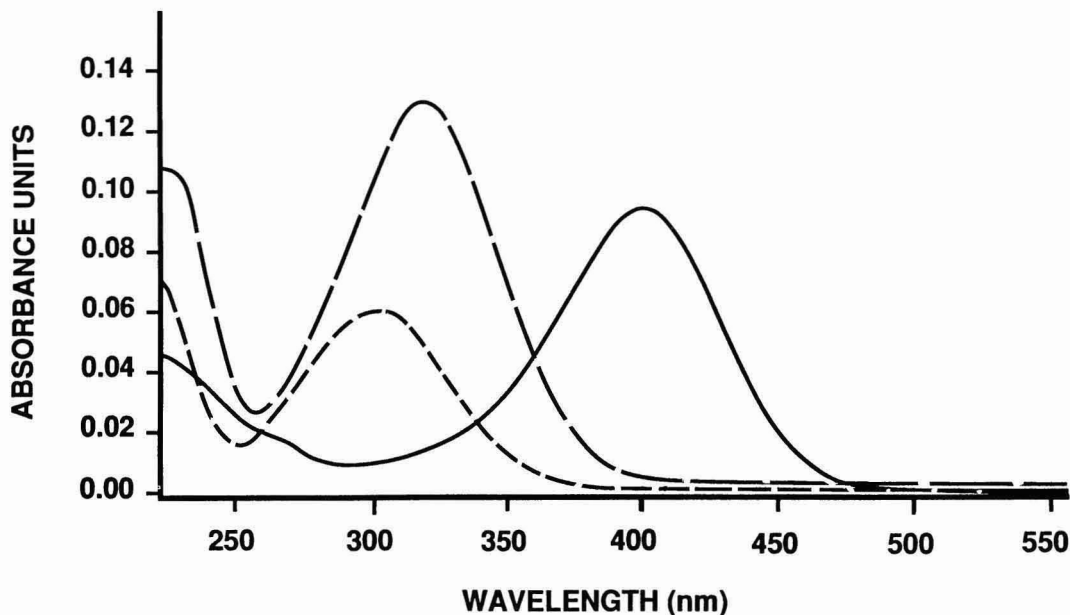


FIGURE 2. Absorption spectra of p-nitroanisole (— — —), p-nitrophenol (————), and p-nitrophenol glucoside (— · — · —). All solutions were 2 mg/l in Instant Ocean®.

ally eluted with 50 ml each of methylene chloride and methanol. The methylene chloride and methanol were combined and rotoevaporated down to ca. 10 ml. This method has been shown to provide greater than 90% recoveries for PNA, PNP, and several conjugates (Foster and Crosby 1986).

The extracts were analyzed on a Beckman Model 110B HPLC system with a C-18 column (Alltech Econosphere, 250 mm × 4.6 mm, 5 μm packing). Two linear gradient solvent systems were used, one to analyze for PNA and PNP (acetonitrile-1% aqueous acetic acid-methanol [60:39:1], going to acetonitrile-1% aqueous acetic acid [95:5] over a period of 10 min), and a second to detect the more polar conjugates (acetonitrile-1% aqueous acetic acid-methanol [10:89:1] held for 3 min, then going to acetonitrile-1% aqueous acetic acid [75:25] over a period of 15 min). Absorbance was monitored with a variable wavelength detector set at 341 nm. This wavelength was selected because PNA, PNP, and PNP-glucoside all absorb there (Figure 2), although sensitivities were reduced.

Dry, decalcified tissue weights were deter-

mined at the end of the depuration period. Algae were decalcified in 200 ml of 10% acetic acid in distilled water. After 24 hr, the tissues were considered decalcified if the pH was less than 4.5 and no more bubbles were being formed (if the pH was greater than 4.5, the acetic acid solution was replaced and allowed to stand for another 24 hr). The decalcified tissues were then dried overnight at 60°C before weighing. The tissue was not analyzed for chemical residues.

RESULTS AND DISCUSSION

UV absorption (PNA concentration) declined in water containing each of the three species during the 24-hr uptake period (Figure 3), while control concentrations showed little loss. Upon removal to clean water (arrow in Figure 3), the algae released PNA. Conversion of these data to tissue concentrations (Figure 4) provided classical uptake, equilibrium, and depuration curves, especially in *Halimeda* experiment 3 (optional curves are shown). *Porolithon* appeared to absorb as much PNA as

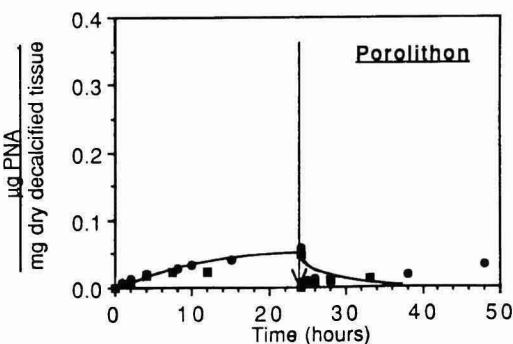
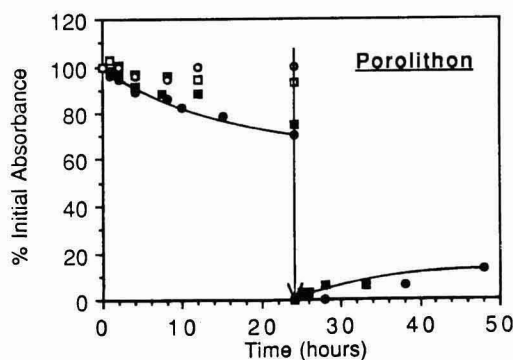
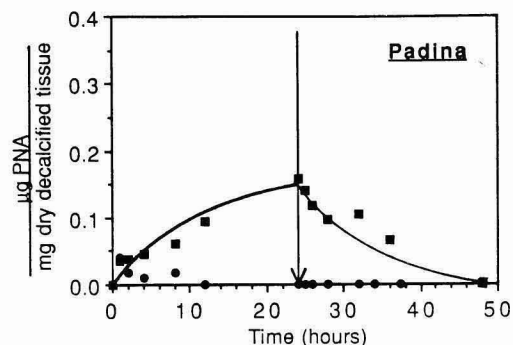
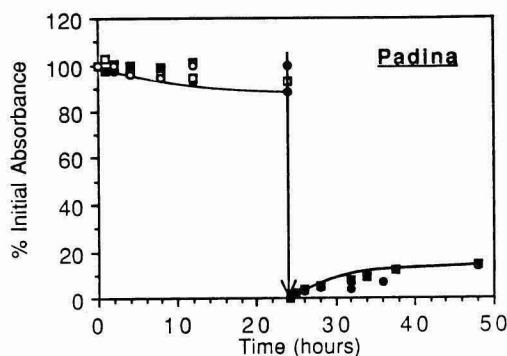
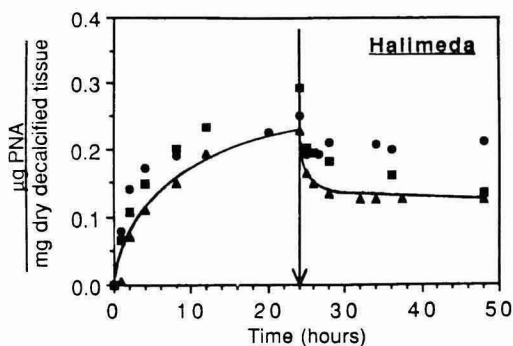
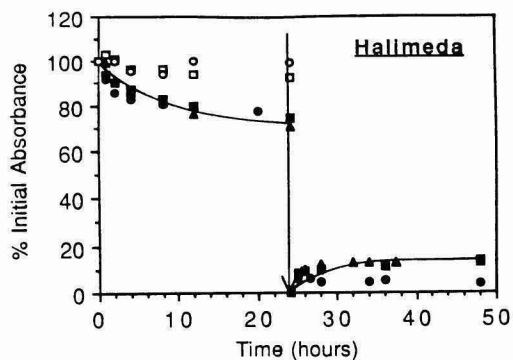


FIGURE 3. Spectrophotometric analysis of PNA in water in the presence (closed symbols) and absence (open symbols) of calcareous algae. Arrow indicates return to clean water. Experiment 1 (●), experiment 2 (■), experiment 3 (▲).

FIGURE 4. Calculated uptake and depuration of PNA in algae. Arrow indicates return to clean water. Experiment 1 (●), experiment 2 (■), experiment 3 (▲).

Halimeda, but upon conversion to tissue concentrations, it actually absorbed much less. This may be a result of incomplete decalcification; *Porolithon* was the most heavily calcified of the three species, and although it appeared to be decalcified using the described method, white particles (calcium carbonate) were observed after drying. The *Padina* results were inconsistent; the first experiment indicated typical uptake and depuration, but in a second experiment, *Padina* did not appear to absorb any PNA.

In each case where the algae were shown to absorb PNA, only about 50% of the absorbed compound was shown to be depurated unchanged. Although trace amounts of the metabolite PNP were detected in some exposures, these levels could not account for the PNA that was absorbed but not depurated. In addition, the presence of PNP was not consistent between replicates and may have been due to PNA metabolism by small invertebrates that still remained after the cleaning process. The PNA may have been retained in the algal tissue or partially bound to particulates in the exposure water that were subsequently filtered out before analysis. Another possibility is that the PNA may have been converted into metabolites that either were not detected with the methods used or were not captured by the XAD-4 resin (p-aminophenol or PNP phosphate). Use of radiolabeled PNA could clarify the matter, but was beyond the scope of this summer project.

Of the three algal species used in these experiments, none absorbed PNP (Figure 5), and there was no HPLC evidence of any metabolism or conjugate formation. However, in a separate experiment, PNP-glucoside was found to be unstable in the 0.2- μm -filtered seawater. Within 24 hr, it was hydrolyzed completely to PNP and glucose. This may also apply to the other conjugates, and if so, none of the PNP conjugates would be detected with the methods used. However, in 0.2- μm -filtered seawater from Bodega Bay, California, the glucoside required 7 days for complete degradation, and it was stable in both distilled water and artificial seawater (Instant Ocean[®]).

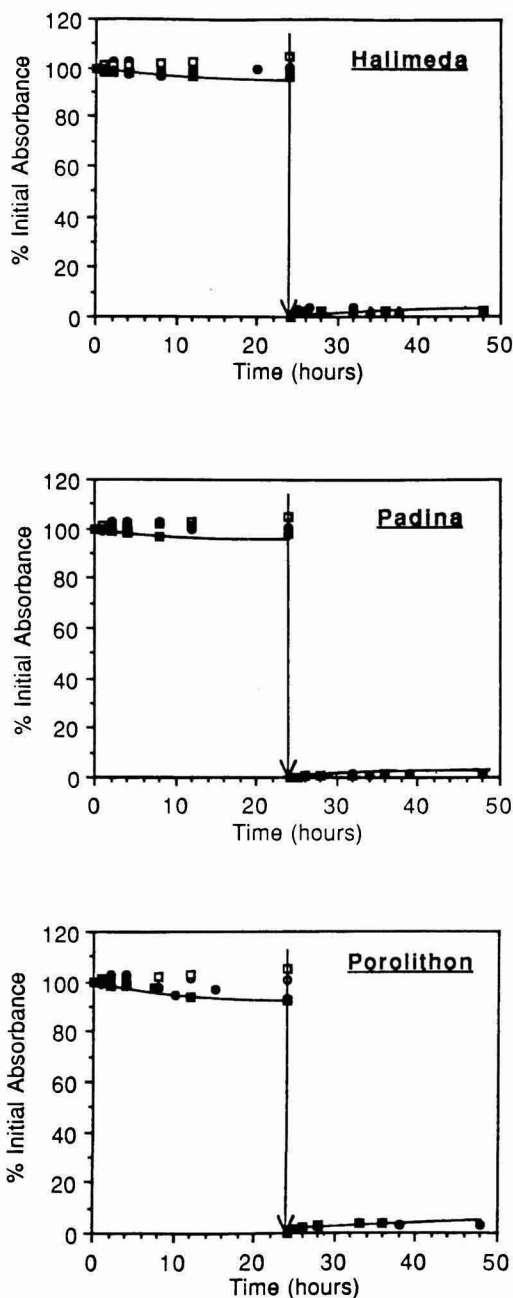


FIGURE 5. Spectrophotometric analysis of PNP in water in the presence (closed symbols) and absence (open symbols) of calcareous algae. Arrow indicates return to clean water. Experiment 1 (●), experiment 2 (■), experiment 3 (▲).

The experiments indicate that these calcareous algae have very little ability to metabolize xenobiotics by the usual routes, and, in addition, they absorb only rather nonpolar compounds. As only half of the absorbed PNA was depurated, either it was metabolized into undetected products or it was retained in the algae, perhaps as PNP bound into tissue macromolecules. If the PNA remained unmetabolized, the algae would concentrate fat-soluble compounds in their tissues, because they are unable to metabolize them into an easily excreted, water-soluble form.

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